TABLE II. Percentage Homology of Nucleotide* and Amino Acid* Sequences of the VP8* Gene of Vietnamese P[6] Strains and Other Human and Porcine P[6] Rotavirus Strains

	M37/Ia	CH9/Ia	ST3/Ia	GR1107/86/Ia	221/04-7/po°/Ib	221/04-13/po/Ib	134/04-10/po/Ic	134/04-11/po/Ic	VN584/2003 Ic	VN592/2003 Ic	VN846/2003 Ic	VN602/2003 Ic	VN904/2003 Ic	51/03/po/Id	Gottfried/po/II	BP720/93 V	BP1198/98 IV	AU19/III
M37/Ia		96.4	97.4	96.2	91.2	91.2	88.2	88.2	88.6	88.4	88.6	88.6	90.0	88.6	77.6	81.8	83.4	79.8
CH9/Ia	92.1		96.4	93.4	88.8	88.8	85.8	85.8	86.8	86.6	86.8	86.8	87.6	86.6	75.6	80.2	81.2	77.4
ST3/Ia	96.7	89.7		96.4	91.0	91.0	87.2	87.2	88.4	88.2	88.4	88.4	89.4	88.6	76.8	81.8	83.2	79.0
GR1107/86/Ia	93.3	87.9	90.9		91.0	91.0	88.2	88.2	90.2	90.0	90.2	90.2	89.6	88.2	77.6	81.4	83.8	79.8
221/04-7/po/Ib	89.7	83.1	87.9	89.7		100.0	90.8	90.8	93.0	92.8	92.6	93.0	93.6	91.6	80.2	85.4	86.6	80.8
221/04-13/po/Ib	89.7	83.1	87.9	89.7	100.0		90.8	90.8	93.0	92.8	92.6	93.0	93.6	91.6	80.2	85.4	86.6	80.8
134/04-10/po/Ic	88.1	82.5	86.7	89.1	94.5	94.5	100.0		94.0	93.8	93.2	94.0	93.8	89.4	79.6	83.8	84.4	80.6
134/04-11/po/Ic	89.1	82.5	86.7	89.1	94.5	94.5	100.0	100.0		93.8	93.2	94.0	93.8	89.4	79.6	83.6	84.4	80.6
VN584/2003/Ic	90.3	84.3	87.9	90.9	96.3	96.3	96.3	96.3	99.3	99.8	98.4	100.0	98.8	89.8	79.4	84.4	85.6	80.6
VN592/2003/Ic	89.7	83.7	87.9	90.3	95.7	95.7	95.7	95.7	100.0	99.3	98.2	99.8	93.6	89.6	79.4	84.2	85.4	80.4
VN846/2003/Ic	90.3	84.3	87.9	90.9	96.3	96.3	96.3	96.3	100.0	99.3	100.0	98.4	93.4	89.8	79.2	83.8	86.2	80.6
VN602/2003/Ic	90.3	84.3	87.9	90.9	96.3	96.3	96.3	96.3	100.0	99.3	100.0	98.4	93.8	89.8	79.4	84.4	85.6	80.6
VN904/2003/Ic	89.1	82.5	86.7	89.1	95.1	95.1	96.3	96.3	96.9	96.3	96.9	96.9	94.5	91.6	80.2	83.6	85.6	80.6
51/03/po/Id	89.7	83.7	87.9	88.5	93.3	93.3	92.1	92.1	94.5	93.9	94.5	94.5	93.3	80.4	83.0	85.8	80.6
Gottfried/po/II	75.9	71.6	74.0	75.3	80.7	80.7	78.3	78.3	80.1	79.5	80.1	80.1	80.1	79.6	77.6	79.8	75.2
BP720/93/V	82.5	78.9	81.3	80.7	87.3	87.3	85.5	85.5	88.5	87.9	88.5	88.5	87.3	86.1		84.4	80.4
BP1198/98/IV	86.1	79.5	84.3	84.3	90.9	90.9	89.1	89.1	92.1	91.5	92.1	92.1	89.7	88.5	80.1	89.1	82.4
AU19/III	80.1	74.0	77.7	79.5	84.9	84.9	85.5	85.5	85.5	84.9	85.5	85.5	86.1	83.7	75.9	84.3	86.7	

Nucleotide homology is shown in the upper right, and amino acid homology is in the lower left. Lineages and sublineages are also indicated.
*Spanning nucleotides 200–700 and amino acids 65–230.
°po, porcine rotavirus.

Fig. 1. Phylogenetic tree of the VP8* fragment of the VP4 gene (nt 200–700) of five Vietnamese and other human and porcine P[6] rotavirus strains. Bootstrap values greater than 75% are indicated at the branch nodes. Vietnamese strains are in bold face. Lineages and sublineages are also shown. The P[19] rotavirus strain Mc345 was used as an outgroup. po, porcine rotavirus.

asymptomatic infection, thus suggesting that the P[6] specificity is associated with virus attenuation [Flores et al., 1986]. In recent studies, however, rotavirus strains with a VP4 genotype [6] similar to that of strains from the avirulent infection described formerly, have been detected from an increasing number of diarrheal infants [Santos et al., 1994; Gentsch et al., 1996]. All five Vietnamese P[6] rotavirus strains in this study were recovered from hospitalized diarrheal patients. Although those patients did not suffer from dehydration, they had a long lasting duration of disease (4–10 days), and a relatively high Vesikari's score (average 9.6/20 points; Table I), clearly indicating the virulence of causative agents.

TABLE III. VP7 Nucleotide and Amino Acid Identities Between Vietnamese G4 and G9 Strains and Other Human and Porcine Rotavirus Strains

Reference strains	G genotype	Species	VN592/2003		VN602/2003		VN846/2003		VN904/2003	
			nt (%)	aa (%)	nt (%)	aa (%)	nt (%)	aa (%)	nt (%)	aa (%)
CMP166	G4	Porcine	98.3	99.0	98.1	99.0	98.1	99.0		
CMP112	G4	Porcine	93.4	98.9	93.2	98.9	93.2	98.9		
Gottfried	G4	Porcine	86.7	94.1	86.8	94.1	86.8	94.1		
GR1106/86	G4	Human	84.4	93.6	84.6	93.6	84.6	93.6		
Hochi	G4	Human	84.3	92.3	84.4	92.3	84.4	92.3		
Odelia	G4	Human	84.3	92.3	84.4	92.3	84.4	92.3		
MR4730/00	G9	Human							99.4	99.3
SI-761/06	G9	Human							98.5	98.7
Mc323	G9	Human							96.4	96.8
A2	G9	Porcine							94.3	96.2
Hokkaido-14	G9	Porcine							92.3	95.5

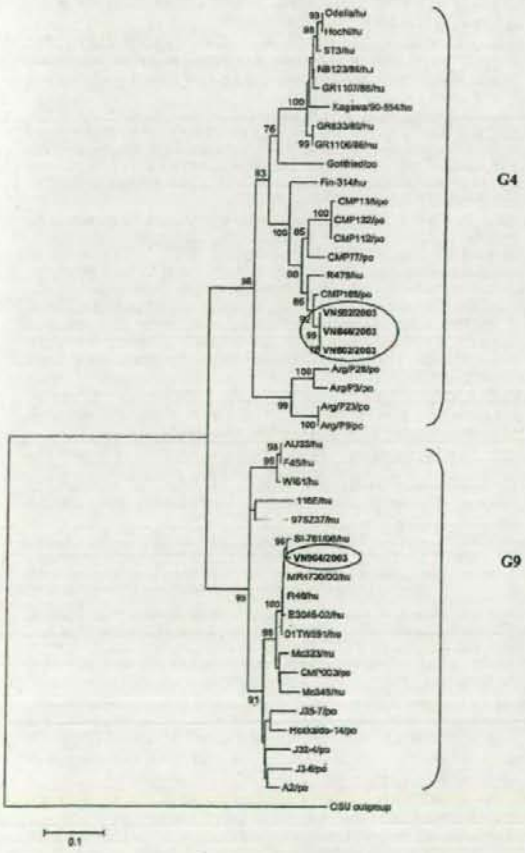

Fig. 2. Phylogenetic tree of the VP7 gene of three Vietnamese G4P[6] and one G9P[6] rotavirus strains. Bootstrap values greater than 75% are indicated at the branch nodes. The Vietnamese strains are in circle. The G5 rotavirus strain, OSU, was used as an outgroup. hu, human rotavirus; po, porcine rotavirus.

The comparison results of sequences of segment 4 from viruses in different serotypes showed that the greatest variation ranges from aa 71 to 204 [Estes, 2001]. Furthermore, Larralde et al. [1991] reported that VP8* possesses the major antigenic sites for serotype specificity within a variable region spanning from aa 92 to 192. Therefore, the submitted amino acid comparison in this study, which spanned from aa 65 to 230, could be adequate to the investigation of characteristics and prediction of serotype specificity of Vietnamese strains. P[6] rotaviruses in different lineages have distinct serotypes: P2A for lineage I; P2B for lineage II; and P2C for lineage III [Li and Gorziglia, 1993; Nakagomi et al., 1999; Banyai et al., 2004] (the lineage IV and V have not been determined yet). Therefore, it is possible to predict that the Vietnamese P[6] strains may share the P2A serotype with other lineage I rotavirus strains.

Although detected in increasing numbers in not only asymptomatic, but also symptomatic children, the majority of human P[6] rotaviruses share high identity and are clustered into a unique lineage (M37-like), except for the "supershort" strain AU19, and recent six unusual strains detected from Hungary that belong to two novel lineages, IV and V. The diversity of lineage I was shown when Martella et al. [2006] introduced Italian and Spanish porcine rotavirus P[6] strains, which were more similar to human P[6]-I strains, and a novel classification of P[6]-I rotaviruses was proposed (Ia–Id). However, the difference between rotaviruses detected from different species was observed, since porcine P[6]-I strains clustered into sublineage Ib–Id, while human P[6]-I strains were highly homogenous and clustered into a unique lineage Ia. Five Vietnamese rotavirus strains in this study, together with other strains (LL36755, KH210, and KH288) are rare rotavirus P[6] strains detected from humans and cluster into lineage Ic with porcine rotaviruses. This result may indicate the common evolutionary origin of the studied strains and other porcine P[6] rotavirus strains. Interestingly, human P[6]-I rotavirus strains that more related to porcine rotavirus were found to be clustered into only a sublineage Ic. Other two sublineages, Ib and Id, composed of only porcine strains.

Recently, many reassortant rotaviruses between human and animal as well as infection of animal viruses in human were reported [Okada et al., 2000; Varghese et al., 2004; Matthijnssens et al., 2006]. Sequence analysis of the VP7 genes of the Vietnamese G9P[6] rotavirus strains, VN904/2003, showed the evidence of a human–animal reassortant virus, with the VP4 gene was closed related to porcine rotavirus, and the VP7 gene had high similarity with human rotavirus. Interestingly, other three G4P[6] strains suggested the infection of animal-like rotavirus in human beings, with the genes encoding for both major neutralizing protein, VP4 and VP7, had the characteristics of porcine rotavirus. Both reference human P[6]-Ic strains that originated from porcine rotaviruses, LL36755 and KH210 [Ahmed et al., 2007; Duan et al., 2007], bear the G5 genotype which is found predominantly in porcine rotaviruses, while Vietnamese animal-like rotavirus strains identified in this study have G4 specificity, which is found widely in both humans and porcine. Regarding patients, all of them did not have any relationship each others. One patient in this study (VN904/2003) lived in Duc Hoa, Long An province, where farming is the main activity, and the other four patients lived on the outskirts of Ho Chi Minh City, where masses of newly built residential districts have been developed, due to waves of provincial immigrants. This information could support the possibility of close contact between humans and animals, thus causing the dynamic interaction of rotaviruses between them.

This study provides the important data on the infection of animal-like rotaviruses in human beings in Vietnam, and highlights the circulation of human P[6] rotavirus strains that have porcine origin in Asian countries.

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Identification of Human Astrovirus Infections Among Children With Acute Gastroenteritis in the Southern Part of Vietnam During 2005–2006

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A molecular epidemiological study on common diarrheal viruses was conducted in a children's hospital in Ho Chi Minh City between December 2005 and November 2006. Fecal samples were collected from 502 pediatric patients with acute gastroenteritis, and were screened for eight types of viral agents. Human astroviruses (HAstVs), which were detected with a prevalence of 13.9%, became the second most frequent viral enteropathogen. Although detected in both dry and rainy seasons, the majority (92.8%) of HAstVs in this study were found in the rainy season. Patients aged between 6 and 23 months were found to be more infected from astrovirus when compared to other age groups. The ratio between boys and girls was 2.3:1. The overall mean severity score of astrovirus positive patients was 11.8 ± 2.45. The mean severity score of the inpatients was statistically higher than that of the outpatients; however, there was no difference in severity scores between the age groups observed. The clinical manifestations of astrovirus infections among the Vietnamese children were diarrhea, vomiting, fever, coughing, and coryza. All of the detected astroviruses belonged to genotype 1. The phylogenetic tree clearly indicated that HAstVs-1 worldwide could be classified into four different lineages, in which, Vietnamese astroviruses and other recently isolated strains from other countries clustered into the distinct lineage, 1d. These results provide important information on astrovirus infections among Vietnamese children. ***J. Med. Virol. 80:298–305, 2008.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: astrovirus; acute gastroenteritis; Vietnam

INTRODUCTION

Human astroviruses (HAstVs) are members of the family *Astroviridae*. They were first identified by electron microscopy in stool specimens from children with diarrhea [Appleton and Higgins, 1975], and recently determined as the second or third most common cause of acute gastroenteritis in infants and young children, with a prevalence varying from 2% to 16.5% [Glass et al., 1996; Gaggero et al., 1998; Mustafa et al., 2000]. The main symptom of astrovirus infection is usually watery and mild diarrhea [Matsui et al., 1993]. Outbreaks of diarrhea associated with astrovirus have been reported in communities, schools, and child care and aged-care center [Glass et al., 1996; Mitchell et al., 1999; Marshall et al., 2007].

HAstV has a positive-sense, single-stranded RNA genome of about 6,800 nucleotide (nt) in length, excluding the poly (A) tail at the 3′ end. Three overlapping open reading frames (ORFs) were determined, in which, ORF1a and ORF1b encode nonstructural proteins, and ORF2 encodes the capsid precursor protein [Willcocks and Carter, 1993; Lewis et al., 1994; Sanchez-Fauquier et al., 1994]. HAstVs are classified into eight serotypes by antigenic reactivity of the capsid proteins [Kurtz and Lee, 1984; Lee and Kurtz, 1994; Taylor et al., 2001], and there

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is a perfect correlation between serotype and genotype [Noel et al., 1995]. Epidemiological studies around the world have demonstrated that serotype 1 is responsible for most astrovirus infections, and serotypes 7 and 8 are rare compared to other serotypes [Lee and Kurtz, 1994; Palombo and Bishop, 1996; Koopmans et al., 1998; Mustafa et al., 2000; Espul et al., 2004]. Recently, molecular analysis of partial nucleotide sequence at the 5′ end of the capsid gene revealed that HAstVs-1 could be classified into four lineages, from 1a to 1d [Guix et al., 2002; Gabbay et al., 2007].

Vietnam is a developing country of 82 million people, in which diarrhea is considered a burden on its young population, and HAstV has been determined as one of the viral agents causing acute gastroenteritis in children [Nguyen et al., 2007]. HAstV infections have been reported in only as sporadic cases in Vietnam [Landaeta et al., 2003; Nguyen et al., 2007]. In this study, we reported the detection of HAstV among diarrhoeic pediatric patients in a Ho Chi Minh City hospital, and described the clinical manifestations of astrovirus-associated acute gastroenteritis as well as analyzing the molecular characteristics of the astrovirus strains detected.

MATERIALS AND METHODS

Surveillance Site and Patients

Children's Hospital 1 is one of two pediatric hospitals in Ho Chi Minh City, with a capacity of 850 beds, receiving pediatric patients from most parts of the city as well as other provinces in the Mekong Delta, South Vietnam. Patients with acute gastroenteritis are screened at the out-clinic ward, and admitted to the Department of Gastroenterology, if necessary. The symptoms of dehydration are assessed by pediatricians, based on the WHO guideline [World Health Organization, 1995], and the severity of diarrhoeic patients is evaluated based on the 20-point numerical score [Ruuska and Vesikari, 1990].

Fecal Sample Collection and Virus Detection

A total of 502 samples were collected from pediatric patients, who visited the hospital from December 2005 to November 2006 with a clinical diagnosis of acute gastroenteritis. The fecal specimens from the outpatients were collected at the out-clinic ward or from the inpatients within 24 hr after admission and stored at −20°C until use. They were prepared as a 10% suspension in distilled water and the viral RNA genomes were extracted from the fecal suspension with a QIAamp Viral RNA Mini Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instruction. The presence of eight types of common viral agents in the fecal specimens was determined by RT-multiplex PCR [Yan et al., 2003, 2004]. Briefly, two sets of primers were used: set A for the detection of groups A, B, and C rotavirus, and adenovirus, and set B was for the detection of norovirus GI, GII, sapovirus, and astrovirus. The primer pair used for the detection of HAstV was PreCAP1 (sense, nt −82 to −62, GGACTGCAAAGCAGCTTCGTG) and 82b (anti-sense, nt 637–617, GTGAGCCACCAGCCATCCCT) [Matsui et al., 1998; Sakamoto et al., 2000], amplifying a 719 bp amplicon (start positions of primers were based on the 5′ end of the capsid gene of the prototype H1 strain, accession number L 23513). Neither bacteria nor parasite was screened in this study.

Genotyping of Astroviruses

Astrovirus genotyping was carried out by using RT-PCR analysis of extracted viral RNA, with serotype specific primers for HAstV-1 to -8, as described previously [Sakamoto et al., 2000]. All PCR products were electrophoresed in 2.0% agarose gel, followed by staining with ethidium bromide (0.5 μg/ml), and then visualized under ultraviolet light. For strains that could not be typed by these processes, the genotype was identified further by sequence analysis.

Nucleotide Sequencing and Phylogenetic Analysis

The 719-bp amplicon of selected astrovirus positive samples was subjected to sequencing by using the Big Dye Terminator Cycle Sequencing Kit version 3.1 and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instruction. The primers, PreCAP1 and 82b, were used for sequencing, and generating a partial nucleotide sequence that included a small part of the 3′ end of ORF1b, and approximately a 550 bp part of the 5′ end of ORF2. Since a number of reference HAstV strains had a nucleotide sequence starting from nt 247 of the 5′ end of ORF2, a comparison with a shorter region (300 bp) spanning nt 247–546 and amino acid 83–182 was performed. Multiple sequence alignments were calculated using the CLUSTALX program, and the phylogenetic tree was constructed by the neighbor-joining method with the MEGA3.1 software package [Kumar et al., 2004], based on different astrovirus sequences available from GenBank.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the capsid gene of the selected Vietnamese astroviruses described in this study were deposited into GenBank under accession numbers EU030287–EU030319. The reference strains and accession numbers used for sequence analysis were as follows: A2/88 (Z25771), Arg158 (AY324858), Bcn1.1 (AF348753), Bcn1.3 (AF348755), BrG1-5 (DQ139825), Dresden (AY 720892), H1 (L23513), H2 (L13745), H3 (AF117209), H4 (Z33883), H5 (U15136), H6 (Z46658), H8 (Z66541), JAPAS115 (AB000287), Melb1E (AF175253), Melb1F (AF175254), PA-COD379-BR (DQ917390), PAK437 (AB 000285), PAstV (AB037272), RJ8479/BR (DQ381498), S7 (AB000300), Ven835 (AF211956), and WH2009 (DQ 788612).

Statistical Analysis

SPSS was used for statistical analysis. The *t*-test was used to compare the prevalence rates among different age groups, as well as mean severity scores between the different populations studied. $P < 0.05$ was considered to be significant.

RESULTS

Detection of Target Viruses

Five out of eight target viral agents were detected in 347 of 502 (69.1%) fecal specimens. As expected, group A rotavirus was the most predominant cause, with an overall prevalence of 53.2%. Interestingly, astrovirus was the second most common viral enteropathogen in this study (70/502, 13.9%), followed by norovirus GII, adenovirus, and sapovirus, with an overall prevalence of 6.4%, 2.4%, and 1.2%, respectively.

Regarding monoinfection, group A rotavirus, astrovirus, genogroup II norovirus, adenovirus, and sapovirus were detected in 46.8%, 9.8%, 3.4%, 1.2%, and 0.4% of samples, respectively. Mixed infection between more than one target virus was found in 38 samples (7.6%), in which, coinfection between group A rotavirus and the other viral agents was dominant (Table I).

TABLE I. Distribution of Target Viral Agents Detected From 502 Children With Acute Gastroenteritis in the Children's Hospital 1, Ho Chi Minh City, During 2005–2006

	Monoinfection					Multiple infection									
	RAV	HAstV	NoV II	AdV	SaV	RAV—HAstV	RAV—NoV II	RAV—SaV	RAV—AdV	NoV II—HAstV	AdV—HAstV	NoV II—AdV	NoV II—SaV	RAV—NoV II—HAstV	RAV—AdV—HAstV
No. (%) of specimens	235 (46.8)	49 (9.8)	17 (3.4)	6 (1.2)	2 (0.4)	15 (3.0)	10 (2.0)	3 (0.6)	2 (0.4)	2 (0.4)	2 (0.4)	1 (0.2)	1 (0.2)	1 (0.2)	1 (0.2)

RAV, group A rotavirus; HAstV, human astrovirus; NoV II, norovirus genogroup II; AdV, adenovirus; SaV, sapovirus.

Seasonal Pattern of Human Astrovirus Infections

The southern part of Vietnam, including Ho Chi Minh City, has a tropical climate, in which the dry season usually begins in November and ends in April of the following year. The remaining months are the rainy season. The average temperature and mean monthly relative humidity does not change much during the year. In this study, HAstVs were found in two distinct periods; one being from March to May, which falls during the end of the dry season and beginning of the rainy season, and the other during the rainy season from August to November. However, 65 out of 70 (92.8%) HAstVs detected in this study were identified in the rainy season (Table II). It is noteworthy that the detection rate of HAstV in October was 70%, which was much higher than that of rotavirus—the most common viral agent in this study.

Characteristics of HAstV Positive Patients

Patients who enrolled in this study were classified into five age groups: <6, 6–11, 12–23, 24–35, and >35 months old. Among 502 patients, 477 medical records were available for analysis of the age distribution, in which, 43, 168, 198, 46, and 22 patients belonged to each of the five age groups, respectively. Diarrhea due to HAstV was seen in all five age groups, and astrovirus infections were more likely found in patients 6–11 and 12–23 months old ($P < 0.001$) (Table III). The ratio between boys and girls was 2.3:1. Thirty out of 70 HAstV-positive cases (42.9%) lived in Ho Chi Minh City, and other patients (57.1%) came from 17 provinces in the southern part of Vietnam.

TABLE II. Monthly Distribution of Viral Enterophatogens Detected From Children With Acute Gastroenteritis in the Children's Hospital 1, Ho Chi Minh City, During 2005–2006

	Dry season						Rainy season					Dry	
Seasonality	Dec 2005	Jan 2006	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Total (%)
No. of specimens	20	30	43	57	30	53	32	17	30	32	60	98	502 (100)
RAV (%)	7 (35.0)	15 (50.0)	23 (53.5)	44 (77.2)	24 (80.0)	29 (54.7)	19 (59.4)	9 (52.9)	12 (40.0)	7 (21.9)	16 (26.7)	62 (63.3)	267 (53.2)
HAstV[a] (%)	0 (0)	0 (0)	0 (0)	**1 (1.8)**	**2 (6.6)**	**12 (22.6)**	0 (0)	0 (0)	**7 (23.3)**	**4 (12.5)**	**42 (70.0[b])**	**2 (2.0)**	70 (13.9)
NoV II (%)	2 (10.0)	1 (3.3)	2 (4.7)	4 (7.0)	0 (0)	3 (5.7)	2 (6.3)	0 (0)	6 (20.0)	4 (12.5)	5 (8.3)	3 (3.1)	32 (6.4)
AdV (%)	0 (0)	0 (0)	2 (4.7)	2 (3.5)	0 (0)	3 (5.7)	1 (3.1)	0 (0)	0 (0)	1 (3.1)	3 (5.0)	0 (0)	12 (2.4)
SaV (%)	0 (0)	1 (3.3)	2 (4.7)	0 (0)	1 (3.3)	1 (1.9)	0 (0)	0 (0)	1 (3.3)	0 (0)	0 (0)	0 (0)	6 (1.2)

[a]The data for HAstV are in bold face.
[b]Detection rate of HAstV in October was higher than that of RAV.

TABLE III. Attributes of 70 HAstV Positive Cases and Mean Severity Score of Patients in Each Group

	Distribution of patients by										
	Age (month)					Gender		Place of living		Patient status	
	<6	6–11	12–23	24–35	>35	Male	Female	HCMC[a]	Others	Inpatient	Outpatient
No. (%) of cases[b]	4 (5.7)	27 (38.6[c])	33 (47.1[c])	3 (4.3)	3 (4.3)	49 (70)	21 (30)	30 (42.9)	40 (57.1)	62 (88.6)	8 (11.4)
Vesikari's score[d]	10.8 ± 3.0	11.8 ± 2.7	11.9 ± 2.4	11.5 ± 2.1	11.5 ± 0.5	11.6 ± 2.5	12.1 ± 2.4	11.6 ± 2.5	11.9 ± 2.7	12.2 ± 2.3[e]	9.4 ± 2.2[e]

[a]HCMC, Ho Chi Minh City.
[b]Data based on 70 HAstV positive cases.
[c]$P < 0.001$.
[d]Data based on 44 out of 49 cases, which showed monoinfection with HAstV.
[e]$P < 0.01$.

Clinical Signs and Symptoms of Astrovirus Infections

Among 49 patients showing monoinfection with HAstV, 44 medical records were enough data to analyze the clinical signs and symptoms of astrovirus infections. The main clinical manifestations observed in children with HAstV infection were diarrhea (100%), vomiting (84.1%), highest temperature ≥38.5°C (50.0%), coughing (40.1%), and coryza (25.0%). The majority of patients (79.5%) presented watery stool, whereas 13.6% had mucus and 6.9% mucus and blood in the stool. Only 4 out of 44 (9.9%) patients showed moderate dehydration, while the other patients had no dehydration during the entire course of the disease. Evaluation of severity in patients by using the 20-point numerical score showed that the scores ranged between 6 and 17 points, with the overall mean score of 11.8 ± 2.45.

The severity scores were analyzed further by age groups, gender, place of living, and status of patients (Table III). Obviously, the mean score of patients <6 months of age was lower than that of patients in the other age groups; however, the difference was not statistically significant ($P > 0.05$). Similarly, the mean scores of severity between male and female patients, as well as between patients who lived in Ho Chi Minh City and other provinces were not statistically different. However, the inpatients in this study suffered from a severer disease than the outpatients, with the mean severity scores in each group being 12.1 ± 2.3 and 9.4 ± 2.2, respectively, and the difference was statistically significant ($P < 0.01$).

Genotyping of Astrovirus

Fifty-six out of 70 specimens, which presented positive for HAstV, were successfully typed by nested PCR. All of them showed the band of PCR product at the position of the genotype 1 (212 bp). Then, 14 untypeable samples were subjected to sequencing at the 5′ end of the capsid gene, and a BLAST search clearly showed that they had best identities (99%) with a genotype 1 human astrovirus, Dresden strain. Altogether, all of the 70 HAstVs detected in this study were genotype 1.

Nucleotide Sequence Analysis of Astrovirus

Thirty-three specimens, including 14 untypeable and 19 selected typeable specimens, successfully determined the nucleotide sequences. The phylogenetic tree, based on the 300 bp region of the 5′ end of ORF2, revealed that all of the Vietnamese astroviruses were grouped into the HAstV-1 cluster [Guix et al., 2002]. Furthermore, the phylogenetic tree clearly showed that HAstVs-1 could be classified into four lineages (1a–1d), in which, Vietnamese astrovirus strains clustered into the lineage, 1d, together with other astrovirus strains recently isolated from Spain, Germany, Brazil, and China. Other HAstVs isolated during the early 1990s, including the prototype H1, clustered into the other three lineages, 1a–1c (Fig. 1).

The nucleotide comparison showed that Vietnamese astrovirus strains were 99.0–100% identical to each other. The similarities between Vietnamese strains and other astrovirus strains in the lineage, 1d, were from 97.3% to 99.6%, in which Vietnamese strains and the Dresden strain were most similar. The divergences between Vietnamese strains and other HAstVs from lineage 1a–1c varied from 8.0% to 12.1%. Nucleotide sequence changes resulting in amino acid substitutions in relation to the prototype H1 were observed in only one strain, HCMC201/2006, with two residues TS instead of NP at position aa 109-110. Wider observation of amino acid comparison at the 5′ end of ORF2 revealed that the HCMC220/2006 strain shared a substitution T instead of A at position aa 46 with the PAK437/91 strain. Furthermore, six strains (HCMC169/2006, HCMC201/2006, HCMC215/2006, HCMC216/2006, HCMC225/2006, and HCMC365/2006) shared a unique residue W, instead of R, at position aa 20. Interestingly, all Vietnamese astrovirus strains showed a distinct substitution of S instead of N at position aa 44, which was not found in any other astrovirus strains among genotype 1.

DISCUSSION

In this study, we described a 1-year surveillance of common viral agents causing acute gastroenteritis in children at a pediatric hospital in Ho Chi Minh City. More than half (57.1%) of the patients who enrolled in this surveillance came from various provinces in the Mekong Delta, therefore, the results of this study might reflect the pattern of astrovirus infection in the southern part of Vietnam. Many of the target viruses were detected in concordance with the previous epidemiological study conducted at the same hospital during 2002–2003 [Nguyen et al., 2007], thus confirming the diversity of viral enterophathogens and the burden of viral diarrhea in the area. As expected, group A rotavirus was still the most frequent cause, however, HAstV became the second most common agent with an overall prevalence of 13.9%, which was much higher than that of the previous study, 0.6% [Nguyen et al., 2007]. A similar detection method was used in both studies, confirming the emergence of HAstV during 2005–2006. On the other hand, although detected in both the dry and rainy season, the majority (92.8%) of HAstVs in this study were found in the rainy season. This result was in agreement with the previous study in Vietnam [Nguyen et al., 2007] and other reports from Brazil [Gabbay et al., 2007; Santos et al., 2007]. Furthermore, HAstVs were found at an exclusively high detection rate (70%) in October from patients who lived not only in Ho Chi Minh City, but also various provinces in the Mekong Delta (data not shown), suggesting that the viruses were widespread in the area at that time. Since fecal specimens were collected at the out-clinic ward or within 24 hr after admission, outbreak of nosocomial infection was excluded.

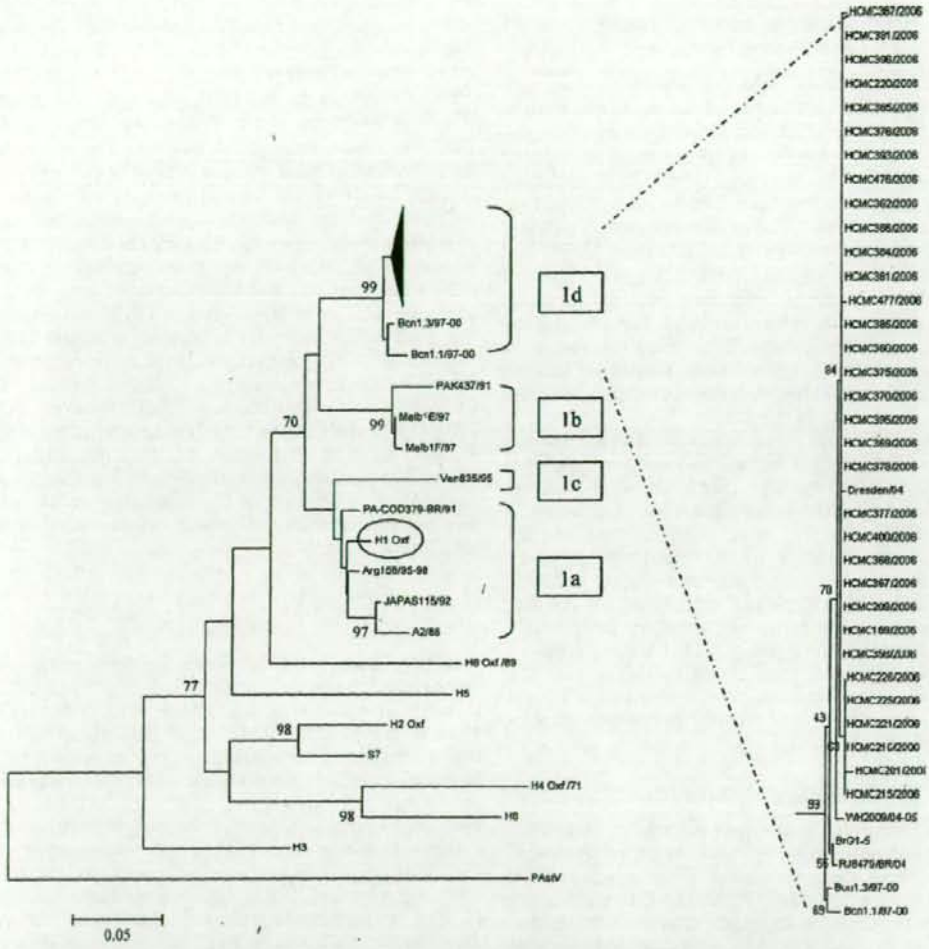

Fig. 1. Phylogenetic tree of the 300 bp region of ORF2 of 33 HAstVs-1 from Ho Chi Minh City (HCMC), and other reference astrovirus strains available from GenBank. The prototype H1 is circled. Four lineages of HAstV-1 are indicated, with the lineage, 1d, including Vietnamese strains and other strains isolated from Spain, Germany, Brazil, and China (Bcn1.1, Bcn1.3, Dresden, BrG1-5, RJ8479/04, and WH2009/04-05). The porcine astrovirus, PAstV, is used as an outgroup.

In this study, HAstVs were found in specimens collected from patients in all five age groups. However, infants aged 6–11 months and children from 12 to 23 months old were found to be infected more than patients in the other age groups. This result was concordant with other astrovirus studies from different countries [Medina et al., 2000; Mustafa et al., 2000; Gabbay et al., 2007]. The protection from HAstV infection might be explained by the passive immunity in infants <6 months, or the active immunity from previously acquired infections in children more than 2 years of age. Interestingly, although the mean severity score of patients <6 months was lower than that of patients in the other age groups, the difference was not statistically significant. This finding suggests that passive immunity only helps to protect from infection event, but does not influence the severity of disease.

The clinical manifestations of HAstV infection in patients in this study were similar to those from other reports; with diarrhea, vomiting, and fever being the main clinical signs and symptoms. However, body temperature ≥38.5°C was found in 50% of patients, and bloody diarrhea was noted in three patients. These features were different from data reported by Walter and Mitchell [2003], in which, only 20–25% of patients had fever, and no bloody diarrhea was reported. On the other hand, although only 9.9% of patients in this study suffered from dehydration, the mean severity score was

relatively high (11.8 ± 2.45), when compared to the mean severity score reported previously (only 5) [Walter and Mitchell, 2003]. This high score might result from a long duration of diarrheal disease, and/or high numbers of diarrhea or vomiting episodes per day (data not shown). However, it is well known that astrovirus can occur in mixed infections with a variety of other infectious agents such as bacteria. Since bacteria and parasite were not screened in this study, the question remains whether HAstV was the sole causative agent that created severity of diarrhea disease among the patients studied.

In agreement with the previous study conducted in Ho Chi Minh City, all of the HAstVs isolated in this study belonged to genotype 1, in which, all of the HAstV were determined as genotype 1 by RT-multiplex PCR. This indicated circulation of genotype 1 HAstVs in the entire area. The predominance of HAstV-1 was also found in all epidemiological studies over the world [Palombo and Bishop, 1996; Medina et al., 2000; Mustafa et al., 2000; Guix et al., 2002; Gabbay et al., 2007], except for one in Mexico, where HAstV-2 was the most common serotype [Guerrero et al., 1998]. On the other hand, Gabbay et al. [2007] reported that the concordance observed between RT-PCR and sequencing in genotyping of astrovirus was only 69.2% due to several reasons, in which, the discordant results were mostly related to HAstVs-4 and HAstVs-7. Our data of 19 typeable HAstV-1 strains, subjected to sequence analysis, indicated that the two typing methods mentioned above were highly agreeable, and it also emphasized the high specificity of the type-specific primer, AST-S1, which was used for genotyping HAstV-1. Data from another study using another set of primers also showed an absolute concordance between results of RT-PCR and sequencing among typeable cases [Mitchell et al., 1999]. Recently, nucleotide sequencing was chosen as the typing method for astrovirus in many studies, thus confirming the usefulness of this method [Mitchell et al., 1999; Medina et al., 2000].

This is the first time that data on nucleotide analysis of Vietnamese astrovirus were reported. In this study, HAstVs-1 could be divided into four lineages, in which, Vietnamese astroviruses and other strains which have been isolated since the end of the 20th century, clustered together into the lineage, 1d. This suggests an evolution of HAstV-1 through nucleotide mutations overtime. Furthermore, the presence of HAstVs-1d from various countries on different continents suggests how widely they spread around the world. Besides, many recent studies have used a 348 bp region of ORF2, generating from primers Mon269 and Mon270 [Noel et al., 1995], for analyzing the nucleotide characteristics of the astrovirus detected [Medina et al., 2000; Mustafa et al., 2000; Guix et al., 2002; Gabbay et al., 2007]. With a longer sequence obtained (719 bp), the primer pair, preCAP1 and 82b, were useful in screening HAstV and performing nucleotide sequence analysis at the capsid region. Within the 300 bp region, spanning nt 247–546, all of the nucleotide mutations within Vietnamese astroviruses were silent, except for one (HCMC201/2006). However, a wider observation toward the 5′ end of ORF2 showed that more substitutions uniquely occurred among Vietnamese astroviruses. This finding confirmed the difference between HAstVs isolated from Ho Chi Minh City and others in other lineages, as well as the prototype H1.

The results of this study highlight the impact of astrovirus in diarrheal diseases among children in the southern part of Vietnam, and are the first to describe the clinical manifestations of astrovirus infections in Vietnamese children. The data of nucleotide analysis from this study could provide useful information for knowledge on astrovirus characteristics, as well as the development of an astrovirus vaccine.

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Short communication

Evaluation of immunochromatography and commercial enzyme-linked immunosorbent assay for rapid detection of norovirus antigen in stool samples

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Abstract

The efficiency of immunochromatography and commercial enzyme-linked immunosorbent assay (ELISA) kit (Denka Seiken Co. Ltd., Tokyo, Japan) were evaluated for rapid detection of norovirus (NoV) from stool specimens. A total of 503 stool specimens collected from infants and young children who suffered from acute gastroenteritis were tested for NoV by the NoV-immunochromatography kit, Denka ELISA kit, and by a monoplex RT-PCR method. The NoV-immunochromatography revealed 78.9% sensitivity, 96.4% specificity, and 92.4% efficiency with the monoplex RT-PCR method. The Denka ELISA kit had a sensitivity of 90.4%, specificity of 96.4%, and an efficiency level of 95%. The findings indicate that the newly developed NoV-immunochromatography kit provides the specificity equal to that of the Denka ELISA kit, even through the sensitivity of detection was lower. However, the advantage of the NoV-immunochromatography kit is less time consuming and simpler. The data show that both the Denka ELISA and the NoV-immunochromatography kits may be used as an alternative method for screening of NoV in stool samples.

Keywords: Norovirus; Immunochromatography; Denka ELISA; RT-PCR; Gastroenteritis

Viral gastroenteritis is one of the most common illnesses in humans worldwide, and different agents such as rotavirus, astrovirus, adenovirus, and calicivirus have been associated with the disease (Clark and McKendrick, 2004). Norovirus (NoV) is one of the four members of the family *Caliciviridae*, which is a nonenveloped, positive-sense, single-stranded RNA virus. NoV is a leading cause of gastroenteritis worldwide and responsible for outbreaks in various epidemiological settings, including restaurants, schools, day-care centers, hospitals, nursing homes, and cruiser (McEvoy et al., 1996; McIntyre et al., 2002; Russo et al., 1997). Currently, based on the diversity of the capsid sequences, NoVs are grouped into five genogroups (G), of which GI, GII, and GIV have been found in humans (Kageyama et al., 2004; Zheng et al., 2006). Human NoV genogroups are subdivided further into at least 15 genotypes in GI, 18 genotypes in GII, and only one genotype in GIV (Kageyama et al., 2004; Okada et al., 2005; Vinje et al., 2004). Several epidemiological studies clearly indicated that NoV GII is the main causative agent among NoVs that cause acute diarrhea in humans (Hansman et al., 2004; Phan et al., 2006a,b; Tseng et al., 2007).

Noroviruses were first discovered by Kapikian et al. (1972) under electron microscopy (EM). More recently, application of reverse transcription-polymerase chain reaction (RT-PCR) and DNA sequencing techniques to detect and characterize NoV became the standard methods for detecting this pathogen. Although RT-PCR is used around the world as a standard tool for routine diagnosis of NoV infection, detection of viral agents with molecular techniques requires well-trained personnel and sophisticated equipments. Thus, a rapid and sensitive diagnostic test for NoV detection is required. Currently, a number

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of enzyme-linked immunosorbent assay (ELISA) kits for the detection of NoV in stool samples have been developed and commercialized (Burton-MacLeod et al., 2004; de Bruin et al., 2006; Richards et al., 2003). In addition to these laboratory diagnostic techniques, the immunochromatography diagnostic test is another alternative choice. The immunochromatography method is easy to perform and less time consuming to conduct the test. In this study, we evaluated the newly developed immunochromatography kit (Immuno-Probe, Co. Ltd., Saitama, Japan) in comparison with a new commercial ELISA kit (NV-AD; Denka Seiken Co. Ltd., Tokyo, Japan) to assess their sensitivities in detecting NoV antigen in stool samples. The monoplex RT-PCR was used as a "gold standard" method for this assessment.

A total of 503 stool samples were collected from infants and children with acute gastroenteritis, encompassing five different geographical settings in Japan (Maizuru, Tokyo, Sapporo, Saga, and Osaka) from July 2004 to March 2005. The presence of NoV GI and GII in fecal specimens was detected by RT-PCR using a protocol described previously (Yan et al., 2003). A forward primer G1-SKF (nt 5342–5261) 5′-CTGCCCGAATTYGTAAATGA-3′ was used in combination with the reverse primer G1-SKR (nt 5653–5671) 5′-CCAACCCARCCATTRTACA-3′, for the amplification of NoV GI. For NoV GII identification, a forward primer COG2F (nt 5003–5028) 5′-CARGARBCNATGTTYAGRTGGATGAG-3′ was used in combination with the reverse primer G2-SKR (nt 5367–5389) 5′-CCRCCNGCATRHCCRTTRTACAT-3′. All of the NoV positive samples were characterized further for their genotypes by direct DNA sequencing using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed the sequence on an automated sequencer (ABI 3100; Applied Biosystems). The sequences obtained were compared to those of NoV strains deposited in the GenBank using the BLAST program, and the genotypes were classified using the clustering determined previously by Kageyama et al. (2004).

To evaluate the sensitivity and specificity of this NoV-immunochromatography test, all of the 503 samples were tested for NoV antigen using the newly developed NoV-immunochromatography kit, which was kindly provided by the Immuno-Probe Company. The immunochromatography test was performed according to the manufacturer's directions. The NoV-immunochromatography strip used in this study was a nitrocellulose membrane coated with gold colloid conjugated with mouse monoclonal antibody (MAb 14-1) against GII/4 on right hand side of the adsorbent pad as shown in Fig. 1. From the previous study, the MAb 14-1 showed a broad range of cross-reactivity with several genotypes of the virus-like particles (VLPs of NoV) as tested by ELISA (Shiota et al., 2007). Therefore, the MAb 14-1 was selected and used as a capture antibody in this NoV-immunochromatography kit. The test line was coated with NoV polyclonal antibodies against NoV GII/3 and GII/4, while the control line was coated with antibody against mouse immunoglobulin. For a negative immunochromatography reaction, only a band of control was appeared on the immunochromatography strip, while a positive immunochromatography reaction both the control and test bands were appeared on the immunochromatography strip (Fig. 1).

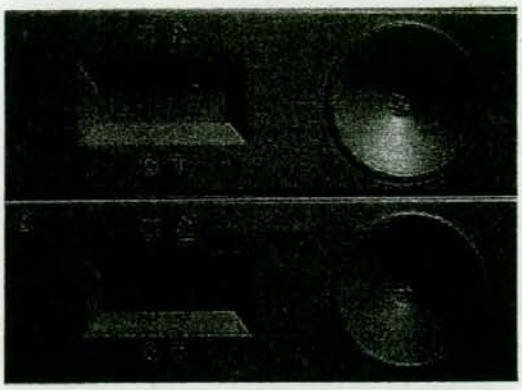

Fig. 1. Detection of NoV in a stool sample using the NoV-immunochromatography kit. The test is negative when only one band appears in the control area (A). The test is positive if two bands appear in the membrane (B). C represents the control band and T represents the test band.

An improved version of commercial ELISA kit, NV-AD (Denka Seiken Co. Ltd.), was evaluated using the same set of stool samples. The detection of NoV using the NV-AD Denka ELISA kit was slightly modified from the manufacturer's instruction. Briefly, 100 μl of a 20% stool suspension was mixed with 120 μl of sample extraction buffer. The reactions for the detection of NoV antigens were performed by mixing 100 μl of the NoV extract with 100 μl of peroxidase conjugated antibodies against NoV GI and GII in the wells which coated with antibodies against NoV GI and GII. After the wells were washed, 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide were added, and a colorimetric reaction was allowed to develop for 30 min at room temperature. The optical density (OD) of the colorimetric reaction was measured at 450 and 630 nm by an ELISA plate reader (Titertek Multiskan, USA). The sample with OD value greater than cut-off value was regarded as a positive reaction.

From a total of 503 fecal specimens collected from pediatric patients with diarrhea, 114 (22.7%) were found to be positive for NoV using the monoplex RT-PCR screening method (Table 1). Of these, majority of the samples (112; 98.2%) belonged to the NoV subgroup II (GII), while only 2 (1.8%) carried subgroup

Table 1
Comparison of NoV detection in stool samples between NoV-immunochromatography kit and commercial ELISA kit (Denka) with monoplex RT-PCR method

Test kit	Monoplex RT-PCR		
	Positive	Negative	Total (%)
Immunochromatography			
Positive	90	14	104 (20.7)
Negative	24	375	399 (79.3)
Denka ELISA			
Positive	103	14	117 (23.3)
Negative	11	375	386 (76.7)
Total (%)	114 (22.7)	389 (77.3)	503 (100)

Table 2
Comparison of the accuracy of the NoV genotype detection between NoV-immunochromatography and ELISA Denka kit with monoplex RT-PCR

Test kit	NoV genotypes determined by monoplex RT-PCR and sequence analysis				
	GI/1	GII/3	GII/4	GII/6	Total (%)
Immunochromatography					
Positive	1	13	75	1	90(78.9)
Negative	1	1	20	2	24(21.1)
Denka ELISA					
Positive	2	12	86	3	103(90.4)
Negative	–	2	9	–	11(9.6)
Total (%)	2(1.8)	14(12.3)	95(83.3)	3(2.6)	114(100)

I (GI) specificity. All of the NoV positive samples were characterized further for identification of their genotypes by sequence analysis of the capsid regions. It was found that both of the GI strains belonged to the GI/1 genotype (1.8%). Of the total 112 GII specimens detected, 95 (83.3%) were GII/4, 14 (12.3%) were GII/3, and 3 (2.6%) were GII/6 genotypes (Table 2).

To evaluate sensitivity, specificity, and efficiency of the newly developed NoV-immunochromatography kit, the results of 503 stool samples tested by NoV-immunochromatography were compared with those of the monoplex RT-PCR method. Of 114 samples that were positive by RT-PCR, 90 were positive by NoV-immunochromatography test, and 24 were negative. In addition, of 389 samples that negative by RT-PCR, 14 were positive by NoV-immunochromatography kit (Table 1). It was interesting to note that although this NoV-immunochromatography kit was developed for the detection of NoV GII/3 and GII/4, which were the major NoV genotypes circulating in humans, a cross-reactivity with some of GI/1 and GII/6 specimens were also observed (Table 2). The sensitivity and specificity of this NoV-immunochromatography test were 78.9 and 96.4%, respectively, and the overall efficiency compared to the monoplex RT-PCR method was 92.4%.

Based on the monoplex RT-PCR standard method, a total of 114 samples were positive for NoV detection. Of these, 103 samples were also positive by the Denka ELISA kit, while 11 samples showed discrepant results. In addition, from the 389 samples that negative by PCR, 14 were positive by the Denka ELISA kit (Table 1). Moreover, it was found that the Denka kit could detect four NoV genotypes (GI/1, GII/3, GII/4, and GII/6 as determined by sequence analysis) (Table 2). Overall, the sensitivity, specificity, and level of efficiency between the Denka ELISA kit and monoplex RT-PCR conventional method were 90.4, 96.4, and 95%, respectively. In comparison of the Denka ELISA assay and immunochromatography kit, the Denka ELISA showed a higher level of sensitivity than the immunochromatography kit (Denka kit: 90.4%, immunochromatography kit: 78.9%). However, specificity of the two kits was equal (96.4%).

Recently, large outbreaks of NoV occurred in various epidemiological settings in Japan. Although the main causative agent of these outbreaks was the GII/4 genotype, other genotypes were also detected and the predominant genotype was changed from one season to others (Morioka et al., 2006; Okada et al., 2005; Sasaki et al., 2006; Tokutake et al., 2006). When patients are diagnosed as severe diarrhea, rapid virus detection is essential for the intervention of appropriate treatment. For this reason, a new NoV-immunochromatography kit was developed to serve as a rapid method for identification of NoV directly from stool samples. In addition to the NoV-immunochromatography kit, the ELISA assay is another attractive supplementary method for screening of NoV in stool samples.

Previously, the sensitivity and specificity of immunochromatography for the detection of NoV in stool samples has been evaluated in our laboratory with a low sensitivity (72.7%) (Okame et al., 2003). The detection of NoV in stool specimens using the commercial RIDASCREEN ELISA kit (R-Biopharm AG, Darmstadt, Germany) was also reported with the sensitivity and specificity of 76.3 and 94.9%, respectively (Okitsu-Negishi et al., 2006). In the present study, the newly developed NoV-immunochromatography kit was evaluated and the results were compared with that of previous NoV-immunochromatography kit. It seems likely that the newly developed NoV-immunochromatography kit shows the sensitivity (78.9%) and specificity (96.4%) higher than that of the previous NoV-immunochromatography kit.

In this study, the new commercial ELISA kit (NV-AD) developed by Denka Seiken Co. Ltd. was also evaluated for sensitivity and specificity by comparing with the monoplex RT-PCR method using an identical set of stool samples that tested by the NoV-immunochromatography kit. It was clearly observed that the new Denka ELISA kit (NV-AD) showed the higher sensitivity (90.4%) than those of the NoV-immunochromatography test (78.9%) and RIDASCREEN ELISA kit (76.3%). When comparing of NoV-immunochromatography and RIDASCREEN ELISA kit, there was no significant difference in sensitivity and specificity. However, it should be pointed out that the advantage of NoV-immunochromatography kit is that it takes only 20 min which is much less time consuming to perform the test compare to 4 h by Denka ELISA and RIDASCREEN ELISA kits.

The detection limit of the NoV-immunochromatography assay, using the standard NoV strains, was found to be approximately 10^8 and 10^7 copies per gram of stool for NoV GII/3 and GII/4, respectively, without cross reaction with other diarrheal viruses (Immuno-Probe Co. Ltd., unpublished data). By using monoplex RT-PCR as a standard method, there were 24 samples that showed false negative results by the NoV-immunochromatography kit (Table 1). The discrepancy might be due to a low viral load in stool specimens or genetic variation that leads to antigenic change of NoV protein and fails to be recognized by MAb as reported by other commercial kits (Burton-MacLeod et al., 2004; de Bruin et al., 2006; Richards et al., 2003). Although the new NoV-immunochromatography kit was developed for the detection of GII/3 and GII/4 genotypes, which are the major genotypes detected in humans, it seems likely that this NoV-immunochromatography kit shows cross-reactivity with some of GI/1 and GII/6 from clinical samples (Table 2). Additionally, several NoV-VLP genotypes were used for testing this NoV-immunochromatography kit to determine a cross-reactivity with other NoV genotypes. It was found

that this NoV-immunochromatography kit could detect other genotypes of GII VLPs, including GII/1, GII/12, GII/13, and GII/14 genotypes. However, cross-reactivity with GI VLPs was not observed. In order to clarify this point, additional testing with several other NoV genotypes from clinical samples is essential.

In conclusion, the present study demonstrated that the NoV-immunochromatography kit or Denka ELISA kit could be used as an alternative method for detecting of NoV in stool specimens and may be practical for screening of NoV during outbreaks of food-borne and person-to-person transmitted gastroenteritis.

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Characterization of a Broadly Reactive Monoclonal Antibody against Norovirus Genogroups I and II: Recognition of a Novel Conformational Epitope▽

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Norovirus, which belongs to the family *Caliciviridae*, is one of the major causes of nonbacterial acute gastroenteritis in the world. The main human noroviruses are of genogroup I (GI) and genogroup II (GII), which were subdivided further into at least 15 and 18 genotypes (GI/1 to GI/15 and GII/1 to GII/18), respectively. The development of immunological diagnosis for norovirus had been hindered by the antigen specificity of the polyclonal antibody. Therefore, several laboratories have produced broadly reactive monoclonal antibodies, which recognize the linear GI and GII cross-reactive epitopes or the conformational GI-specific epitope. In this study, we characterized the novel monoclonal antibody 14-1 (MAb14-1) for further development of the rapid immunochromatography test. Our results demonstrated that MAb14-1 could recognize 15 recombinant virus-like particles (GI/1, 4, 8, and 11 and GII/1 to 7 and 12 to 15) and showed weak affinity to the virus-like particle of GI/3. This recognition range is the broadest of the existing monoclonal antibodies. The epitope for MAb14-1 was identified by fragment, sequence, structural, and mutational analyses. Both terminal antigenic regions (amino acid positions 418 to 426 and 526 to 534) on the C-terminal P1 domain formed the conformational epitope and were in the proximity of the insertion region (positions 427 to 525). These regions contained six amino acids responsible for antigenicity that were conserved among genogroup(s), genus, and *Caliciviridae*. This epitope mapping explained the broad reactivity and different titers among GI and GII. To our knowledge, we are the first group to identify the GI and GII cross-reactive monoclonal antibody, which recognizes the novel conformational epitope. From these data, MAb14-1 could be used further to develop immunochromatography.

Norovirus is the major cause of nonbacterial epidemic gastroenteritis (11) and belongs to the family *Caliciviridae* containing five distinct genera, *Vesivirus*, *Lagovirus*, *Norovirus*, *Sapovirus*, and *Becovirus* (33). Norovirus has been identified as the cause of 73% to more than 95% of gastroenteritis outbreaks in the United States and approximately half of those worldwide (1).

Norovirus is classified into five genogroups (genogroup I [GI] to genogroup V [GV]) by genetic diversity: viruses in genogroups I, II, and IV (GI, GII, and GIV, respectively) are associated with diarrhea in humans, with GII also able to infect pigs; genogroups III and V (GIII and GV) are associated with bovines and mice, respectively (19). Moreover, norovirus GI and GII are the main causative agents in humans and subdivided further into at least 15 and 18 genotypes (GI/1 to GI/15 and GII/1 to GII/18), respectively (30).

Because the lack of a cell culture system for norovirus has hindered immunological and structural study, the recombinant virus-like particles (rVLPs), which are morphologically and antigenically similar to native norovirus virions, were expressed by using the baculovirus expression system (12, 16, 37).

Norovirus is composed of 180 molecules (90 dimers) of the single major capsid protein, VP1, which has two principal domains. One is the shell (S) domain, which is highly conserved among animal caliciviruses. The other is the protruding (P) domain, which is divided into three subdomains: N-terminal P1, P2, and C-terminal P1 domains. The P2 domain is the most protruding and diverse domain (37). In addition, the internally located N-terminal domain participates in a network of interactions through domain swapping to assist the assembly of the shell domain into an icosahedral scaffold (6).

Several laboratories have generated polyclonal antibodies by using recombinant VP1 as antigens. The rabbit anti-rVLP polyclonal antibody was highly specific for genotypes used as immunogens (13, 18, 21). This specificity has hindered the development of immunological diagnosis. We previously developed the immunochromatography test for detection of norovirus infection by using the anti-rVLP polyclonal antibody (31); however, this method showed the immunogen's genotype specificity.

Monoclonal antibodies are a useful tool for detecting various kinds of noroviruses, and they are more stable than polyclonal antibodies for use in a rapid immunological assay. The previously reported broadly reactive monoclonal antibodies

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TABLE 1. Capsid fragment primers

Primer[a]	Sequence (5′ to 3′)[b]	Position (nucleotide)[c]	Polarity
1207cacc-418	CAC CGC TCC TGC CGT TGC CCC C	1252–1270	Sense
1207cacc-427	CAC CGG TGA GCA ACT TCT TTT C	1279–1296	Sense
1207-528	CTA TGT GTA GAA CTG GTT GAC CC	1553–1575	Antisense
1207-534	CTA CCC CGC TCC ATT TCC CAT	1582–1602	Antisense
1207-541	GGG CCA TTA TAA CGC ACG TC	1604–1623	Antisense

[a] The numbering of the sense primers indicates the nucleotide sequences of the N-terminal (first) norovirus residue contained within a construct generated with a particular primer. The numbering of the antisense primers indicates the nucleotide sequences of the C-terminal (last) norovirus residue contained within a construct generated with a particular primer.

[b] Four bases used for directional cloning are shown underlined.

[c] The 1207 sequence was assigned accession number DQ975270 in GenBank.

could be classified into two groups by their epitope properties. The first group recognizes the intergenogroup cross-reactive linear epitopes on the S or P domain, NS14, 1B4, and 1F6 (20, 35, 46, 47). The other group recognizes the intragenogroup cross-reactive conformational epitopes, NV3901 and NV3912 (35, 46). In addition, gaining information about the location of norovirus-specific epitopes is essential for designing diagnostic tools (i.e., enzyme-linked immunosorbent assay [ELISA] and immunochromatography), identifying the neutralizing epitope, and developing antivirals and an effective vaccine.

In this study, we describe characterization of a novel monoclonal antibody, which shows broad reactivity with both GI and GII norovirus rVLPs. These findings could be applied for further development of the rapid immunochromatography test, because immunochromatography using this novel antibody has demonstrated high performance in detecting norovirus infection (28).

MATERIALS AND METHODS

Antigens (rVLPs). Sixteen rVLPs were previously expressed by the baculovirus expression system and confirmed by electron microscopy (31, 32). The sequences were genetically classified based on the method described by Kageyama et al. (17). Within GI, five genotypes of rVLPs were generated, including genotypes 1 (strain 4656 [sequence accession number EF547392]), 3 (strain 3634 [EF547393]), 4 (strain 2876 [EF547394]), 8 (strain 3006 [EF547395]), and 11 (strain 2258 [EF547396]). For GII, 11 genotypes of rVLPs were generated, including genotypes 1 (strain 3101 [EF547397]), 2 (strain 2840 [EF547398]), 3 (strain 3229 [EF547399]), 4 (strain 1207 [DQ975270]), 5 (strain 3611 [EF5473400]), 6 (strain 3612 [EF5473401]), 7 (strain 419 [EF5473402]), 12 (strain 2087 [EF5473403]), 13 (strain 3385 [EF5473404]), 14 (strain 2468 [EF5473405]), and 15 (strain 3625 [EF5473406]).

Production of monoclonal antibody. The P363-Ag-U1 myeloma cell line was used as the parent cell. CsCl-purified GII/4 rVLP (r1207) was used as an immunogen for preparing the monoclonal antibody, as previously described (22).

ELISA for titration of the monoclonal antibody. Plates with 96 wells (Maxisorp; Nunc, Roskilde, Denmark) were coated with 90 ng of rVLP/well in 60 μl of 0.1 M carbonate buffer (pH 9.6) for 1 h at 37°C. To compare the reactivities of ELISAs with different pHs, two coating buffer solutions with different pH conditions were used. Phosphate-buffered saline (PBS) with a pH of 7.4 was used, and carbonate buffer with a pH of 9.6 was used only for GII/3 rVLP r3229 and GII/4 rVLP r1207. The wells were blocked with 1% bovine serum albumin in PBS containing 0.1% Tween 20 (PBS-T). The plates were incubated overnight at 4°C. After the wells were washed three times with PBS-T, for titration of the monoclonal antibody, 60 μl of a twofold serial dilution was added to each well, starting with a 1:100 dilution of the monoclonal antibody in PBS-T containing 1% bovine serum albumin, and the plate was incubated for 1 h at 37°C. After the wells were washed three times with PBS-T, 60 μl of a 1:4,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Biosource International, Camarillo, CA) was allowed to react for 1 h at 37°C as the second antibody. After the wells were washed three times with PBS-T, 60 μl of substrate *o*-phenylenediamine containing 0.012% H_2O_2 and 0.2 M citrate-phosphate buffer (pH 5.0) were added to each well and left in the dark for 20 min at room temperature. The reaction was stopped by adding 60 μl of 2 M H_2SO_4 to each well, and the optical density at 492 nm (OD_{492}) was determined (using OD_{650} as the reference) with a Labsystems Multiskan MCC microplate reader (Thermo Electron Corporation, Waltham, MA). For this experiment, Tn5 cell lysate was included as a negative control. A sample that which had an OD of ≥0.2 and signal/noise ratio of ≥2.0, was considered positive. Each assay was conducted in duplicate.

Fragment construction. The pET 100 directional TOPO vector (Invitrogen Corp., Carlsbad, CA) was used to express the capsid fragments with a His tag. The primers used in this study are shown in Table 1. PCR-amplified fragments of r1207 were generated using the primer pairs indicated by the names of the constructs. The template used for the PCR was the previously reported plasmid containing the complete capsid sequence of r1207 (31). PCR fragments were directly cloned into the pET 100 directional TOPO vector. The plasmids were transformed into *Escherichia coli* One Shot TOP10 (Invitrogen Corp., Carlsbad, CA). Positive transformants were identified by PCR. The plasmids from positive transformants were transformed further into *E. coli* BL21 Star cells (Invitrogen Corp., Carlsbad, CA). To express the r1207 capsid fragments, overnight cultures of *E. coli* BL21 cells, transformed with each plasmid, were diluted to a ratio of 1:20 in fresh Luria-Bertani broth supplemented with 100 μg/ml of ampicillin. The cells were grown at 37°C until the culture reached a certain cell density (when the OD_{600} was 0.5 to 0.7). Expression was induced by adding 1.0 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen Corp., Carlsbad, CA), and cultures were grown for an additional 3 h. The cells were pelleted by centrifugation for 15 min at 3,000 × *g* at 4°C. The supernatant was removed, and the cell pellet was suspended in a 1/20 volume of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and a protease inhibitor cocktail [complete, Mini, EDTA-free] [1 tablet/10 ml] [Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany]) and gently shaken at 4°C for 30 min. Following that treatment, Triton X-100 and lysozyme were added to concentrations of 1% and 0.2 mg/ml, respectively, and gently shaken at 4°C for 20 min. Finally, the cells were centrifuged for 30 min at 12,000 × *g* at 4°C, after which the protein was found in the insoluble fraction.

This fraction was resuspended in 20 mM of Tris (pH 8), 500 mM of NaCl, and 8 M of urea, filtered, and loaded onto a HisTrap column (GE Healthcare Bio-Science Corp., Piscataway, NJ) equilibrated in 20 mM of Tris (pH 7.4), 500 mM of NaCl, and 8 M of urea. On-column renaturing was performed with 8 to 0 M urea gradient solutions. The elution was performed with a 0 to 1 M imidazole gradient. The peak fractions were pooled, and the solvent displaced PBS (pH 7.4) from the PD10 column (GE Healthcare Bio-Science Corp., Piscataway, NJ).

Fragment analysis. Analysis of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done by the method of Laemmli et al. (24) with slight modifications. Briefly, 15% polyacrylamide resolving gels and a 5% acrylamide stacking gel were used. Capsid fragments were suspended in electrophoresis sample buffer containing 4% sodium dodecyl sulfate, 10% mercaptoethanol, 125 mM of Tris-HCl (pH 6.8), 0.01% bromophenol blue, and 10% glycerol. Samples were boiled for 5 min. Separated proteins were transferred onto a 0.45 μm polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) in a semidry transfer (CB-09A; ATTO, Tokyo, Japan) at a constant current of 2 mA/cm^2 for 30 min. The blotted membrane was washed with PBS-T and blocked with 5% skim milk in PBS-T overnight at 4°C. The membrane was washed with PBS-T and then incubated overnight at 4°C with an antibody against the five-histidine tag (QIAGEN, Hilden, Germany) and antinorovirus monoclonal antibody diluted to 1/10,000 and 1/1,000, respectively, with 0.5% skim milk in PBS-T. The blot was washed with PBS-T and incubated with a 1/10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Tago, Burlingame, CA). The blot was then reacted with peroxidase substrate solution (diaminoben-

TABLE 2. Site-directed mutagenesis primers

Primer	Sequence (5′ to 3′)[a]	Position (nucleotide)[b]	Polarity
F425G	CTG CCG TTG CCC CCA CTG GCC CGG GTG A	1256–1283	Sense
F425G antisense	TCA CCC GGG CCA GTG GGG GCA ACG GCA G	1256–1283	Antisense
P(CCG)426F(GCG)	CGT TGC CCC CAC TTT CGC GGG TGA GCA ACT TCT TTT C	1260–1296	Sense
P(CGG)426F(CGC)	GAA AAG AAG TTG CTC ACC CGC GAA AGT GGG GGC AAC G	1260–1296	Antisense
L(CTT)526A(GCT)	GGT CAA CCA GTT CTA CAC AGC TGC CCC CAT GGG AAA TGG	1557–1595	Sense
L(AAG)526A(AGC)	CCA TTT CCC ATG GGG GCA GCT GTG TAG AAC TGG TTG ACC	1557–1595	Antisense
A(GCC)527K(AAG)	GGT CAA CCA GTT CTA CAC ACT TAA GCC CAT GGG AAA TGG AGC	1557–1598	Sense
A(GGC)527K(CTT)	GCT CCA TTT CCC ATG GGC TTA AGT GTG TAG AAC TGG TTG ACC	1557–1598	Antisense
P(CCC)528A(GCC)	CCA GTT CTA CAC ACT TGC CGC CAT GGG AAA TGG AGC G	1563–1599	Sense
P(GGG)528A(GGC)	CGC TCC ATT TCC CAT GGC GGC AAG TGT GTA GAA CTG G	1563–1599	Antisense
G(GGA)530A(GCA)	CAC ACT TGC CCC CAT GGC AAA TGC AGC GGG GTA GAA *GG*	1572–1602	Sense
G(TCC)530A(TGC)	CCT TCT ACC CCG CTG CAT TTG CCA TGG GGG CAA GTG TG	1572–1602	Antisense

[a] Mutant nucleotides are shown in boldface type, and vector nucleotides are shown in italic type.
[b] The 1207 sequence was assigned accession number DQ975270 in GenBank.

zidine; SIGMA, St. Louis, MO) to detect the antigen-antibody complexes on the blot.

Sequence analysis. The ClustalX multiple-sequence alignment program (version 1.83) was used for multiple alignment of constructed rVLP sequences and other genogroups (40). The capsid subdomains were determined based on previously reported data from Prasad et al. (37).

Mutational analysis. Specific residues in the capsid fragment, 418 to 534, were altered using the QuikChange XLII site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Some mutagenesis primers were engineered by the QuikChange Primer Design Program (Stratagene, La Jolla, CA) as shown in Table 2. Generated mutants were purified and analyzed by using the same protocols as those for fragment construction and analysis. Mutant clones were confirmed by sequencing.

Structural analysis. The crystal structure of the prototype Norwalk virus capsid protein (PDB code 1IHM) was used to built homology models for r1207 (37). The initial sequence-to-structure alignments and the refined three-dimensional models of r1207 with minimized side chain conformations were obtained using the T-Coffee and SWISS MODEL (29, 38). The figures were made by using PYMOL (http://pymol.sourceforge.net).

Nucleotide sequence accession numbers. Newly determined sequences were submitted to GenBank under accession numbers DQ975270 and EF547392 through EF547406.

RESULTS

Cross-reactivity of the novel monoclonal antibody. The ELISA comparison of the reactivities of the novel monoclonal antibody by using different pH conditions for the coating buffer (pH 7.4 and 9.6) showed that a high pH condition (pH 9.6) could not affect the result of ELISA. The novel monoclonal antibody (MAb14-1) obtained from a mouse immunized with r1207 (GII/4 rVLP) showed broad reactivity against various genotypes of rVLPs by ELISA (Table 3). All the different rVLP norovirus genotypes (GI/1, 3, 4, 8, 11 and GII/1 to 7 and 12 to 15) used in this study were recognized by MAb14-1. However, only a weak affinity to the GI/3 genotype was observed (data not shown). The titers of MAb14-1 were almost the same as those against GII rVLPs, and quite different from those against GI rVLPs (Table 3).

Minimal binding region on the capsid with monoclonal antibody MAb14-1. To determine the binding domain of the VP1 capsid region against MAb14-1, five fragments were constructed with the His tag: full VP1 (amino acid positions 1 to 541), full VP1 except for the N-terminal subdomain (positions 46 to 541), P domain (positions 222 to 541), P domain except for the C-terminal P1 domain (positions 222 to 417), and N-terminal P1 domain (positions 222 to 275). Only fragments not containing the C-terminal P1 domain showed nonreactivity for MAb14-1 (Fig. 1B,. top five schematic fragments). This result suggested that the C-terminal P1 domain might contain the specific epitope of MAb14-1.

In addition, to determine the minimal binding region of MAb14-1, five capsid fragments were constructed by deletion of both terminal regions of the C-terminal P1 domain. It was found that MAb14-1 showed predictable reactivity for the C-terminal P1 domain, while the N-terminal deletion (amino acid positions 418 to 426 from 418 to 541) induced abolition of reactivity for MAb14-1 and the C-terminal deletion mutants with amino acids 418 to 534, 418 to 528, and 418 to 525 deleted induced rise, decline, and abolition of antigenicity, respectively (Fig. 1A and B, bottom five schematic fragments). These results implied that the minimal binding region is probably from amino acid positions 418 to 534 (Fig. 1) and suggested that nine amino acid residues (positions 418 to 426 [A region]) on the N-terminal domain, and three amino acids (526 to 528 [B region]) and six amino acids (529 to 534 [C region]) on the C-terminal domain were important regions for the antigenicity of MAb14-1.

Epitope for monoclonal antibody 14-1. Alignment of the minimal binding regions on rVLPs and other genogroups

TABLE 3. Titers of newly developed MAb14-1 with various rVLPs by ELISA

Monoclonal antibody	Isotype	Titer for rVLP (100)[a]															
		Genogroup I genotype					Genogroup II genotype										
		1	3	4	8	11	1	2	3	4	5	6	7	12	13	14	15
MAb14-1	IgG1	512	<1	8	64	16	8,192	2,048	4,096	**8,192**	2,048	4,096	4,096	1,024	8,192	2,048	2,048

[a] The homologous titer is shown in boldface type.

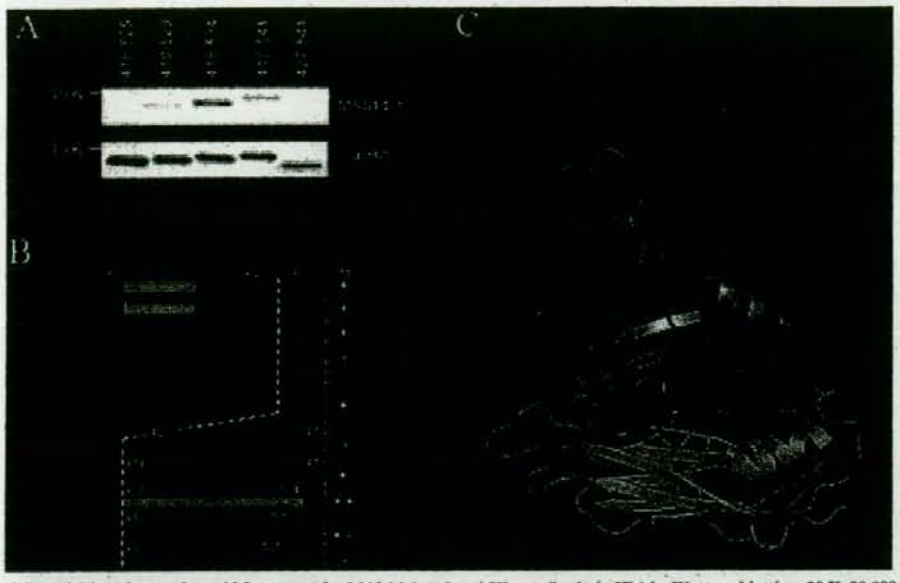

FIG. 1. (A) Reactivities of several capsid fragments for MAb14-1 and anti-His_5 antibody (a-His) by Western blotting. 20 K, 20,000. (B) Process of mapping the minimal binding region shown in silver on the map of VP1. WB, Western blotting. Symbols; ++, increase in antigenicity; +, same antigenicity as for r1207; +−, decline in antigenicity; −, abolition of antigenicity. (C) Prediction structure of r1207 (a part of the C-terminal P1 domain prediction structure could not be created through lack of structural data from 1IHM [Protein Data Bank identification code for Norwalk virus capsid protein]). The N-terminal domain (amino acid positions 0 to 45) (green), S domain (positions 46 to 221) (yellow), N-terminal and C-terminal P1 domains (positions 222 to 275 and 418 to 541) (red), and P2 domain (positions 276 to 417) (blue) are indicated in panels B and C.

showed that the deleted terminal regions had genus-specific residues (such as A418 and P419) and genogroup-specific residues (such as V421 and F425), but these regions did not have GI/3-specific single point mutations (Fig. 2).

To confirm the relationship between both terminal regions of highly conserved residues (amino acid positions 418 to 426 [A region] and 526 to 534 [B and C regions]) and antigenicity, genus-specific and GII-specific residues were changed to alanine and GI-specific amino acids, respectively (Fig. 2). The six mutations induced several kinds of changes in antigenicity, whereas other mutations did not affect antigenicity. These effects were classified into four different groups: (i) disappearance of the antigenicity (point mutation of L526A within the B region), (ii) severely attenuated antigenicity (F425G in the A region), (iii) significant reduction in reactivity (P426F in the A region, A527K in the B region, and P528A in the C region), and (iv) moderate reduction (G530A in the C region) (Fig. 3). These results confirmed that three regions affected antigenicity as predicted by alignment analysis.

To confirm that the relationship between these amino acids affected the antigenicity and structure of r1207, prediction of antigen structure was performed by using the registered Norwalk virus (GI/1) capsid structure (Fig. 1C and 3B). This showed that antigenic residues were contiguous with each other (Fig. 3B). The insert region from amino acid positions 427 to 525 made both the terminal regions proximate; however, they did not have direct interactions via charged residues. The interaction between each terminal region and the insert region (positions 427 to 525) was not observed except for hydrophilic interaction. Moreover, a GI/3-specific single point mutation, close to both terminal regions, was not identified in our study (Fig. 2).

DISCUSSION

In this study, a newly developed monoclonal antibody (MAb14-1) was identified as being a broadly reactive monoclonal antibody, which recognized GI (GI/1, 4, 8, and 11) and GII (GII/1 to 7 and 12 to 15) rVLPs with a weak affinity to GI/3. This recognition range is the broadest of the existing monoclonal antibodies (Table 4) (14, 20, 35, 39, 46, 47). MAb14-1 shows low affinity to GI, but this result was also observed in a previous report by Yoda et al. (46). Therefore, we determined the broad reactivity of MAb14-1 after Yoda's observation.

In this study, the coating buffer with pH 9.6 was used for the antibody titration. Previous and recent studies repeatedly showed that rVLPs disassemble completely into soluble capsid proteins when a pH value is equal to or higher than 8.9; therefore, rVLPs no longer exist at pH 9.6 (2, 45). From this observation, the same experiments using a coating buffer with pH 7.4 were performed only for r3229 and r1207; however, different conditions showed the same results. Therefore, the data on titer and broad reactivity of the antibody can be compared with not only several laboratory results using rVLPs but also with results using recombinant capsids (or fragments) (Table 4). To explain this reactivity at the molecular level, we demonstrated precise determination of the epitope by using